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ABSTRACT

Women who are carriers of inherited germline mutations have an 85% lifetime risk of developing breast cancer. BRCA1 carriers are more likely to develop tumors at an earlier age that are estrogen receptor negative, therefore they cannot benefit from antiestrogen chemopreventive treatments. Because currently it is not possible to predict who will actually develop breast cancer, we have designed studies using molecular approaches for identifying the "high risk" genomic signature of the cytological normal breast epithelium of women at high risk for breast cancer. This signature will serve as an intermediate biomarker for evaluating the response of the breast to novel chemopreventive agents. We performed cDNA microarray analysis of pure epithelial cell populations obtained by laser capture microdissection (LCM) from cytological smears of the normal human breast epithelial cell line MCF-10F. Our observations confirmed the usefulness of combining LCM and cytospin preparations for obtaining pure cell populations for RNA extraction, and of PCR RNA amplification for cDNA microarray analysis and quantification of gene expression level by real time RT-PCR. These studies will lead to fruitful results through genomic hierarchical cluster analysis and Bioinformatics for patient risk assessment and for evaluating the responsiveness of the breast epithelium to chemopreventive agents.

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Introduction

Breast cancer, the most frequently diagnosed malignant disease in American and Northern European women of all ethnic groups, is continually increasing in incidence in most industrialized countries (1-3). Early detection and improved treatment modalities have succeeded in reducing breast cancer mortality rates in the United States by 2.3% per year since 1990 (3,4). However, it is fatal once it metastasizes, remaining as the number one cause of cancer-related death in non-smokers (1). Although the cause of the disease and of the worldwide increase in incidence remains largely unknown, the main risk factors that predispose the breast to malignancy have been clearly identified to be endogenous, namely, being of female sex, having a functional ovary cyclically secreting estrogens, exhibiting specific endocrinological conditions, such as early menarche, late menopause, and nulliparity (5-7), and being carriers of inherited germline mutations (8-10). The risk of these populations is further increased by exposure to environmental factors such as radiation (11,12), circadian disruption acting as endocrine disrupter (13,14), smoking (15,16), and alcohol consumption (17). Among the endogenous factors, the inheritance of germline mutations in the *BRCA1* or *BRCA2* genes is low in frequency, affecting less than 5% of the female population (8-10). However, it represents a definitive threat, since women that are carriers of these mutations are at an 85% lifetime risk of developing breast cancer. The disease is also diagnosed at a significantly younger age than sporadic breast cancer, and the tumors, especially in women carriers of the mutant *BRCA1,* are more aggressive and generally negative for estrogen (ER) and progesterone (PR) receptors, therefore they cannot reap the benefits of antiestrogen preventive and therapeutic modalities (18-20). Thus, the only preventive measure available to this high-risk population is prophylactic mastectomy (10,20). This dismal picture emphasizes the need of preventing this disease as the ultimate approach for its control. Recent progress in defining the early premalignant phases of breast cancer and the development of molecular tools have provided novel biomarkers that will contribute to making primary prevention a real possibility (21). The identification in preclinical studies that mammary cancer is prevented if the mammary gland becomes differentiated prior to its exposure to a carcinogenic agent (22-32) led us to postulate that hormonally induced differentiation is the most physiological approach for the prevention of cancer in women. Towards this goal we have designed experiments for determining whether the protection conferred by the hormones of pregnancy and more specifically, human chorionic gonadotropin (hCG) protect the mammary gland through the induction of a specific genomic signature that characterizes the differentiated mammary gland (33,34). This signature, in turn, would serve as an intermediate biomarker for assessing the response of the mammary gland to specific hormonal stimuli and as a predictor of resistance to neoplastic transformation.

Body

We have demonstrated that RNA extracted from the mammary gland of virgin Sprague-Dawley rats that have received a daily intraperitoneal injection of 100 IU/hCG for 21 days after analysis employing an oligonucleotide microarray provides a gene expression profile that is characteristic for this hormone. The genomic signature induced by hCG is similar to that induced by pregnancy, and is characterized by upregulation of genes that are *activators or repressors of transcription,* i.e., CREB1, p21, and GADD153, a*poptosis;* i.e., caspase 1; *growth factors,* i.e.,

TGF-beta3; *cell division control,* i.e. p21 Cdk 1 inhibitor; *DNA repair genes,* i.e., GADD153 and Casein kinase delta; *tumor suppressor genes*, i.e., p53, *Cell-surface antigens*, i.e., B7.1, CD-28, and CD44 antigen. The levels of gene expression have been validated by real time RT-PCR, confirming that the mammary glands of hCG-treated virgin rats and of parous rats, which are resistant to chemically induced carcinogenesis, exhibit permanent genomic changes that serve as intermediate biomarkers of cancer prevention (33-35).

Our studies have further demonstrated that hCG treatment of rats previously exposed to DMBA inhibits the progression of preneoplastic lesions, such as intraductal proliferations and carcinomas in situ (29,35-39). This indication of a direct effect of hCG on the mammary epithelium has been further confirmed by *in vitro* hCG treatment of MCF-10 and MCF- 7 cells, normal and neoplastic breast epithelial cell lines respectively, In these cells, hCG inhibits growth, depresses cell proliferation, lengthens the G1 phase of the cell cycle, and induces the synthesis of α and β inhibin, a non-steroidal glycoprotein belonging to the TGB-β family with demonstrated tumor suppressor activity (40,41). These observations indicate that hCG acts both through ovarian stimulation and directly on individual mammary cells to inhibit cancer cell growth by activating an autocrine/paracrine loop mediated by inhibin.

Based on the strength of the preclinical data described above, a pilot clinical trial was conducted to determine whether hCG administered to breast cancer patients would induce in the tumors the same biomarkers identified in the rodent mammary cancers and that are indicators of cancer regression (42). Twenty-five postmenopausal women with palpable primary breast cancers>1.5 cm were eligible. After confirmation of the diagnosis by core biopsy, patients were randomized to receive 500 μg of recombinant human chorionic gonadotropin (r-hCG) by intramuscular injection every other day times seven, or saline placebo. At the completion of the study treatment, all patients underwent mastectomy. Pre- and post-treatment tumor specimens were examined for rate of cell proliferation, estrogen and progesterone receptor expression, and immunoreactivity for inhibin. Treatment with r-hCG induced a statistically significant decrease in rates of cell proliferation and down regulation of ER and PR expression in tumor cells. At the same time, a significant increase in immunoreactivity for inhibin was observed in the neoplastic cells. No systemic changes in ovarian steroid or pituitary hormones were observed, suggesting that the effects of hCG are organ specific (42). Thus, our experimental *in vitro* and *in vivo* systems have demonstrated that hCG exerts both a preventive effects by inducing a specific genomic signature as result of mammary gland differentiation that prevents the initiation of chemically-induced mammary carcinomas and the induction of differentiation of chemically transformed cells, inhibiting the progression of mammary carcinomas.

Human chorionic gonadotropin (hCG) has been used clinically for many years for the treatment of male and female infertility, corpus luteum insufficiency, habitual or threatened abortion, hypogonadism and cryptorchidism in the male, and weight reduction (43-45). It is well tolerated without significant toxicities and therefore offers enormous promise as a chemopreventive agent for women with a familial/genetic risk for breast cancer. However, the inability to predict in this high risk population who will develop breast cancer has required the implementation of broad, population-based strategies utilizing preventive measures that have significant side effects and require protracted treatment. Therefore, we are addressing the need of precisely identifying those women who should take a preventive agent, sparing others who will not develop the disease

during their lifetimes. It is in this status of knowledge that we have developed a new paradigm for breast cancer prevention, the identification of the genomic and proteomic signatures that characterize the breast at high risk. The accomplishment of this goal became feasible due to the combination of nipple fluid aspirate (NFA) with the more recently developed ductal lavage (46-49), a noninvasive procedure that yields both cellular and non-cellular material for evaluation. Cellular and breast fluids obtained using these methodologies provide material adequate for analysis utilizing high-throughput microarray (50,51) and proteomic technologies (52-54). The selection of pure epithelial cell populations by laser capture microdissection (LCM) allows researchers the amplification of RNA from well characterized cells in which cytopathological and immunocytochemical characteristics that have served as traditional biomarkers can be correlated with novel genomic and proteomic signatures. These combined technologies represent powerful tools for generating a vast amount of information on the genomic and protein profiles of the breast ductal system in a single experiment. While working under this training grant we have analyzed pure epithelial cell populations obtained by laser capture microdissection (LCM) utilizing cytological smears of the normal human breast epithelial cell line MCF-10F, a normal immortalized human breast epithelial cell line that has been maintained in our laboratory for more than 14 years (55). The first objective was the determination of the minimal number of cells that would yield adequate quantity of high quality RNA for genomic analysis.

Cell Culture: MCF-10F cells were grown in DMEM/F12 high-calcium (1.05 mM) medium at 37°C in a humidified atmosphere of 5% CO2. When the cultures reached 80-90% of confluence, the cells were trypsinized, counted using hemacytometer and fixed in nuclease free 50% ETOH for 20 minutes.

Cytospin preparation: Cell suspensions were evenly distributed in 8 cytospin non adhesive slides and centrifuged at 1200 rpm at room temperature for 10 min. Each slide contained approximately 4.6 x 105cells. After centrifugation slides were dried at room temperature for 2 min, sequentially followed by immersion in 75% ETOH for 30 sec, ddH_20 for 30 sec, Histostain for 20 sec, ddH_20 for 30 sec, 75% ETOH for 30 sec, 95 % ETOH for 30sec, 100% ETOH for 30 sec, xylene for 5 min, and air dried for 5 min.

Laser Capture microdisection (LCM): Three set of cells were collected in duplicate using LCM: 20, 200 and 2000 cells. LCM was performed using the AutoPix 1000 Laser capture equipment (Arcturus, CA). The microdisection conditions were: Power; 70 uV, laser diameter: 15 μm. The cells captured were placed in nucleic lysis solution and kept on ice until processed.

RNA isolation: Total RNA from each sample was extracted with Acturus PicoPure RNA Isolation Kit. Cells were mixed with 20 ul of RNA extraction buffer and incubated at 42°C for 30 min, processing thereafter following the manufacturer's recommended procedures. All purified RNA samples were DNAsed and measured by Nanodrop and quality was assessed by Bioanalyzer 2100 (Agilent Technologies, Inc, CA) (Table 1).

RNA amplification: Two methodologies for RNA amplification were applied to each sample, Linear RNA amplification and PCR-based RNA amplification procedures. Both procedures are based on oligo-dT-T7 primer amplification followed by in vitro-transcription reaction (IVT) (Table 1).

Table 1. Total RNA extracted from MCF-10F cells after LCM

MCF10F Cells Number	Initial Total RNA (ng)	Total Volume (µl)[1]	Amplified aRNA UV260/UV280	Concentration (ng/µl)
20	NA	10	1.35	NA
20	NA	10	1.33	NA
200	NA	10	1.35	NA
200	NA	10	1.37	NA
2000	21	10	1.94	2.1
2000	65	10	1.90	6.5

[1]PicoPure Extracted TRNA with 1 µl out of 10 µl total RNA

RT-PCR: Real Time RT-PCR was used for validating the data obtained from each sample. The housekeeping gene 18S was utilized for generating a standard curve. Concentrations of 18S gene were compared with those of the breast epithelium specific genes fat milk globule membrane antigen and whey acidic protein (Table 2).

Table 2. 18 S RT-PCR Measurement in Total RNA

MCF10F Cells Number	Initial Total RNA (ng)	Total Volume (µl)[1]	RT-PCR Volume (µl)	Concentration (ng/µl)
20	0.33	10	1	0.033
20	0.38	10	1	0.038
200	0.35	10	1	0.035
200	0.73	10	1	0.073
2000	19	10	1	1.9
2000	53	10	1	5.3

[1]with 1 µl out of 10 µl total RNA

Our results led us to conclude that RT-PCR is sensitive for detecting RNA from small numbers of LCM selected cells, being able to detect few copies of RNA, whereas the amounts of RNA obtained by linear amplification did not suffice for its use in microarray analysis. These observations confirmed the usefulness of the application of LCM to cytospin preparations for obtaining pure cell populations for RNA extraction and of PCR RNA amplification for cDNA microarray analysis and of RT-PCR for gene expression level quantification. The possibilities of using a minimal number of cells and the utilization of RT-PCR instead of linear amplification for obtaining RNA in adequate amount and quality for performing cDNA microarray analysis represented a significant step to warrant that the breast epithelial cells from selected donors can

be studied in the same fashion. These studies will lead to fruitful results through genomic hierarchical cluster analysis and bioinformatics for patient risk assessment. This meeting offers a great opportunity to scientists and health care providers of discussing with consumers and the general public affected by breast cancer the potential of this approach for assessing risk using molecular biomarkers.

Key Research Accomplishments

- Confirmation of the usefulness of the application of laser capture microdissection (LCM) to cytospin preparations for obtaining pure cell populations for RNA extraction
- Confirmation of the usefulness of RT-PCR RNA amplification for cDNA microarray analysis
- Confirmation of the usefulness of RT-PCR for gene expression level quantification.

Reportable Outcomes

This study has allowed us to confirm the possibilities of using a minimal number of cells (20) and the utilization of RT-PCR instead of linear amplification for obtaining RNA in adequate amount and quality for performing cDNA microarray analysis. This step was significantly important to warrant that the breast epithelial cells from selected donors can be studied in the same fashion.

Russo, I.H., Wang, R., Balogh, GA, Sheriff, F. Masny, A., Daly, MB, Torosian, M., Russo, J. IDENTIFICATION OF MOLECULAR BIOMARKERS OF CANCER RISK IN CYTOLOGICALLY NORMAL BREAST EPITHELIAL CELLS. Era of Hope meeting in Philadelphia, Pennsylvania, June 8-11, 2005. (attached in the appendix)

Conclusions

The goal of this application, the identification of genomic signatures that might be indicative of a high risk for cancer development of women who are carriers of BRCA/BRCA2 mutations, was accomplished through the utilization of molecular approaches for characterizing patterns of gene expression in cytologically normal breast epithelium. Our studies have confirmed the advantages of combining the use of laser capture microdissection (LCM) for capturing specific numbers of pure epithelial cell populations for RNA extraction, PCR amplification, and cDNA microarray analysis, as well as for confirmation of the level of gene expression by RT-PCR quantification. Our observations led us to conclude that the utilization of pure cell populations for applying genomic hierarchical cluster analysis and bioinformatics will serve in the future for patient risk assessment and for evaluating the response of the breast epithelium to chemopreventive or therapeutic agents.

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Appendix

Russo, I.H., Wang, R., Balogh, GA, Sheriff, F. Masny, A., Daly, MB, Torosian, M., Russo, J. IDENTIFICATION OF MOLECULAR BIOMARKERS OF CANCER RISK IN CYTOLOGICALLY NORMAL BREAST EPITHELIAL CELLS. Era of Hope meeting in Philadelphia, Pennsylvania, June 8-11, 2005.

Appendix

Russo, I.H., Wang, R., Balogh, GA, Sheriff, F. Masny, A., Daly, MB, Torosian, M., Russo, J. IDENTIFICATION OF MOLECULAR BIOMARKERS OF CANCER RISK IN CYTOLOGICALLY NORMAL BREAST EPITHELIAL CELLS. Era of Hope meeting in Philadelphia, Pennsylvania, June 8-11, 2005.

Women who are carriers of BRCA/BRCA2 mutations may have a lifetime risk of breast cancer as high as 85%. Even though it is known that BRCA1 mutation carriers are more likely to develop tumors that are estrogen receptor negative, being less likely to benefit from antiestrogen chemopreventive treatments, currently it is not possible to predict who will actually develop breast cancer. The use of molecular approaches will allow us to identify the "high risk" genomic signature of the cytological normal breast epithelium of women at high risk for breast cancer. This signature will serve as an intermediate biomarker for evaluating the response of the breast to novel chemopreventive agents.

For this purpose we analyzed pure epithelial cell populations obtained by laser capture microdissection (LCM) utilizing cytological smears of the normal human breast epithelial cell line MCF-10F. We captured 20, 200, and 2000 cells. RNA from each set of cells was extracted with a commercially available RNA isolation kit. Cells were mixed with 20 µl of RNA extraction buffer and incubated at 42°C for 30 min. Purified RNA was measured by fluorometric quantitation and a linear RT-PCR assay developed in our laboratory. In order to obtain 20-40 µg RNA needed for microarray analysis, RNA was amplified using linear and PCR amplification. The housekeeping gene 18S was utilized for generating a standard curve. Concentrations of 18S gene were compared with those of the breast epithelium specific genes fat milk globule membrane antigen and whey acidic protein.

Our results led us to conclude that RT-PCR is sensitive for detecting RNA from small numbers of LCM selected cells, being able to detect few copies of RNA, whereas the amounts of RNA obtained by linear amplification did not suffice for its use in microarray analysis. These observations confirmed the usefulness of the application of LCM to cytospin preparations for obtaining pure cell populations for RNA extraction and of PCR RNA amplification for cDNA microarray analysis and of RT-PCR for gene expression level quantification. The possibilities of using a minimal number of cells and the utilization of RT-PCR instead of linear amplification for obtaining RNA in adequate amount and quality for performing cDNA microarray analysis represented a significant step to warrant that the breast epithelial cells from selected donors can be studied in the same fashion. These studies will lead to fruitful results through genomic hierarchical cluster analysis and bioinformatics for patient risk assessment. This meeting offers a great opportunity to scientists and health care providers of discussing with consumers and the general public affected by breast cancer the potential of this approach for assessing risk using molecular biomarkers.